(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2001 (27.09.2001)

PCT

(10) International Publication Number
WO 01/71011 A1

(51) International Patent Classification[7]: C12N 15/74 // C12R 1/225

(21) International Application Number: PCT/EP00/03099

(22) International Filing Date: 23 March 2000 (23.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant *(for all designated States except US)*: **INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE** [FR/FR]; 147, rue de l'Université, F-75338 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GORY, Laure** [FR/FR]; Dempeyre, F-43700 Coubon (FR). **MONTEL, Marie-Christine** [FR/FR]; 14, rue Gray Bouliagua, F-15000 Aurillac (FR). **ZAGOREC, Monique** [FR/FR]; 86, rue Charles de Gaulle, F-78350 Jouy En Josas (FR).

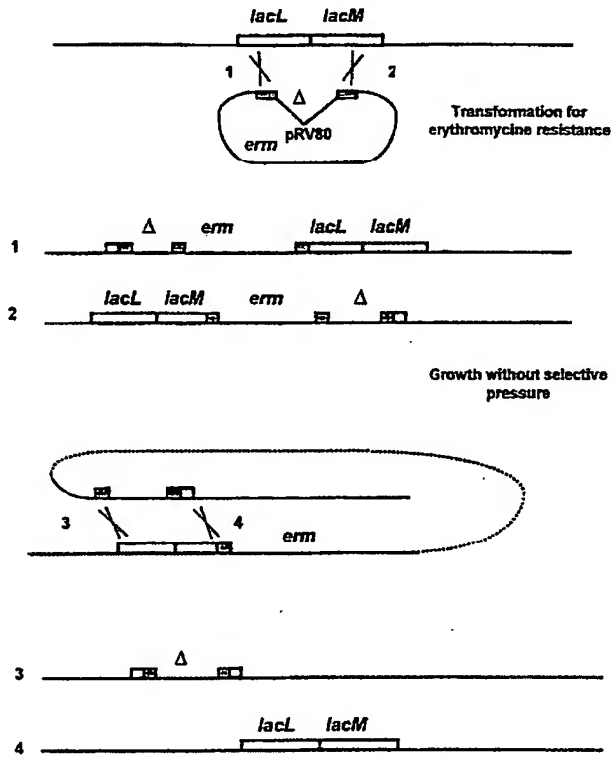
(74) Agents: **VIALLE-PRESLES, Marie-José** et al.; Cabinet Ores, 6, avenue de Messine, F-75008 Paris (FR).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN; YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent

[Continued on next page]

(54) Title: VECTORS FOR THE GENETIC TRANSFORMATION OF LACTOBACILLUS SAKEI



(57) Abstract: The invention concerns methods and vectors allowing to obtain stable transformants of *L. sakei*. More specifically, the invention provides integrative vectors allowing insertion of heterologous DNA at the *LacLM* chromosomal locus. The invention also concerns marker genes for monitoring *L. sakei* strains.

WO 01/71011 A1

(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:
— *with international search report*

VECTORS FOR THE GENETIC TRANSFORMATION OF LACTOBACILLUS SAKEI

The invention relates to methods and tools for the genetic transformation of *Lactobacillus sakei.*

Lactobacillus sakei is widely used in the food industry, and more specifically in the production of fermented meat products.

Some *Lactobacillus sakei* strains inoculated as starter cultures play an important role in the development of the organoleptic qualities of fermented meat products. *L. sakei*, by producing lactic acid, contributes to the hygienic safety and sensorial properties of dry sausage and may also have a role in flavor development [HAMMES *et al.*, FEMS Microbiology Reviews, 87, 165-174, (1990)]. However, the strains, which are inoculated as starters, are in competition with the natural flora of meat and fermented meat products composed of *L. sakei* and other *Lactobacillus*, *Staphylococcus* and *Enterococcus* species. Then, in the industry of fermented meat products, there is clearly a need to monitor, among the natural flora, the growth and the activity of inoculated starter cultures. Traditional techniques for microbial assessment lacked the specificity to monitor specific species. Therefore, molecular techniques, such as the use of DNA probes have recently been developed as specific methods to identify and quantify populations of microorganisms in complex environments. *In situ* hybridization with oligonucleotidic probes targeting rRNAs have successfully been used to detect and identify Gram-negative filamentous bacteria in sludge [WAGNER *et al.*, Systematic and Applied Microbiology, 17, 405-417, (1994)], lactococci in milk [BEIMFOHR *et al.*, Systematic and Applied Microbiology, 16, 450-456, (1993)] or sulfate reducing bacteria in mixed cultures AMANN *et al.* [Applied and Environmental Microbiology, 56, 1919-1925, (1990)]. GRANT *et al.* [Journal of Applied Bacteriology, 74, 260-267, (1993)] and FURRER *et al.*, [Journal of Applied Bacteriology, 70, 372-379, (1991)] have detected bacteria in food or cheese by the amplification of specific sequences using the Polymerase Chain Reaction (PCR).

These molecular techniques are species specific and sensitive but do not allow the detection of specific strains. Furthermore, they are limited by nucleic acid extraction from the complex environment and by factors limiting PCR efficiency.

Stable marker systems with an easily detectable phenotype provide an alternative strategy to detect microorganisms in complex environments. Several genes have thus been used as reporter genes in lactic acid bacteria, such as the luciferase genes in *Lactococcus lactis* [CORTHIER *et al.*, Applied and Environmental Microbiology, 64, 2721-2722, (1998); STEWART and WILLIAMS, Journal of General Microbiology, 138, 1289-1300, (1992); EATON *et al.*, J. Gen. Microbiol., 139, 1495-1501, (1993)], the β-glucuronidase (*gusA*) gene in *L. lactis*, *Leuconostoc*

lactis, *Lactobacillus plantarum* and *Lactobacillus casei* [PLATTEEUW *et al.*, Appl. Environ. Microbiol., 60, 587-593, (1994)], the *nuc* gene in *L. lactis* [LE LOIR *et al.*, J. Bacteriol., 176, 5135-5139, (1994)], or the *gfp* gene in *L. plantarum* [GEOFFROY *et al.*, Appl. Environ. Microbiol., 66, 383-391, (2000)] and *L. lactis* [SCOTT *et al.*, FEMS Microbiol. Lett., 182, 23-27, (2000)]. The *lacZ* gene of *Escherichia coli*, encoding β-galactosidase, and the chloramphenicol acetyl transferase gene have also been widely used in bacteria.

However, although several genes have now been cloned from *L. sakei* [HAMMES and HERTEL, Meat Science, 49, 125-138, (1998)] and some genetic tools are emerging [AXELSSON *et al.*, FEMS Microbiol. Lett., 168, 137-143, (1998); BERTHIER *et al.*, Microbiology (Reading), 142, 1273-1279, (1996); LANGELLA *et al.*, FEMS Microbiol. Lett., 139, 51-56, (1996); the molecular biology techniques specific for this species are still poorly developed. For example, no reporter gene system easy to handle has yet been developed that would help in the analysis of gene regulation.

The purpose of the invention is to develop new tools for the genetic engineering of *L. sakei*, and more specifically tools allowing the monitoring of one or several *L. sakei* strain(s) in a complex environment, without altering their growth and activity of *L. sakei*.

Accordingly, the invention provides vectors allowing a stable and strong expression of heterologous DNA sequence in *L. sakei* without interfering with the expression of endogenous genes.

The inventors have now found that surprisingly, derivatives of the pG+host plasmids which have been initially developed as thermosensitive vectors to generate chromosomal insertions in several Gram-positive bacteria [BISWAS *et al.*, Journal of Bacteriology, 175, 3628-3635, (1993); PCT WO 93/18164, CNCM I-1179) are very stable in a replicative form in *L. sakei*, both in growth laboratory conditions and during experimental dry sausage production. An object of the invention is the use of said derivatives of pG+host plasmids as extrachromosomal vectors for the stable expression of a gene of interest in *L. sakei*.

"Derivatives of pG+host plasmids" are herein defined as plasmids comprising at least the thermosensitive replication sequences of the plasmid pVE6002 (CNCM I-1179) disclosed in PCT WO 93/18164.

Further, the inventors have constructed an integrative vector allowing the stable insertion of an heterologous DNA sequence into the chromosomal *lacLM* operon, encoding *L. sakei* β-galactosidase.

The invention relates to said integrative vector resulting from the insertion of a portion of the *lacLM* operon of *L. sakei* comprising of at least 300 pb of

the 5'-end of *lacL* and at least 300 pb of the 3'-end of *lacM* of *L. sakei* into the suicide vector pRV300. pRV300 is disclosed by LELOUP *et al.* [Appl. Environm. Microbiol., 63, 2117-2123, (1997)]. It is composed of a pBluescript SK replicon for propagation in *Escherichia coli* and an erythromycin resistance marker.

An heterologous DNA sequence may be inserted between the *lacL* and the *lacM* sequences.

The invention also relates to a process for obtaining a stable transformant of *L. sakei* wherein said process comprises transforming a *L. sakei* host cell with a vector selected among:

- an integrative vector as defined above,
- a derivative of a pG+host plasmid.

The invention also encompasses *L. sakei* transformants resulting from the transformation of a *L. sakei* host cell with a vector of the invention. This include *L. sakei* cells containing at least one copy of a derivative of a pG+host plasmid as defined above, as well as *L. sakei* cells wherein a part of the chromosomal *lacLM* operon is deleted, or *L. sakei* cells having an heterologous DNA sequence inserted into the chromosomal *lacLM* operon.

The invention also provides marker genes able to be expressed in *L. sakei*, and easily detected even in a complex environment.

In preliminary experiments, the inventors tested several genes commonly used as reporter genes in lactic acid bacteria; however, no satisfactory expression was observed in *L. sakei*. For instance, the luciferase genes could not be expressed at a sufficient level, and the expression of *gusA* could not be detected.

The inventors performed further experimentations, using *lacZ* gene of *E. coli* as a reporter gene. A strain was constructed in which an internal part of the *lacLM* operon was deleted. A construct comprising *lacZ* under transcriptional control of an inducible *L. sake* promoter was inserted into the chromose. Under conditions allowing the induction of the promoter, β-galactosidase activity was detected in the transformed strains, showing that *lacZ* can be used as a reporter gene in *L. sakei*.

However the use of *lacZ* as a reporter gene necessitates that the host cell has no endogenous β-galactosidase activity or that the endogenous β-galactosidase is inactivated by the insertion of the *lacZ* construct into *lacLM*.

In order to obtain a more versatile system, also usable in cells having an endogenous β-galactosidase activity, the inventors tested other reporter genes, and selected the green fluorescent protein (GFP). They constructed *L. sakei* strains comprising a *gfp* gene under the control of a strong constitutive promoter integrated into a replicative plasmid or into the chromosome of *L. sakei*. In both case they observed a strong and stable expression of GFP. Expression of GFP did not alter the

growth of the *L. sakei* transformants and allowed to detect GFP-marked strains directly on plates or in dry sausage. Further, strains obtained with a replicative plasmid or by chromosomal integration were stable both in growth laboratory conditions and during experimental dry sausage production.

Thus, another object of the invention is the use of a gene selected among:

- a gene encoding a β-galactosidase, and preferably the *lacZ* gene encoding the β-galactosidase of *E. coli*, and
- a gene encoding the green fluorescent protein

as a reporter gene in *L. sakei*.

More specifically the invention provides a process for providing a detectable cell of *L. sakei*, wherein said process comprises providing an expression vector comprising a reporter gene selected among:

- a gene encoding a β-galactosidase, and preferably the *lacZ* gene encoding the β-galactosidase of *E. coli*, and
- a gene encoding the green fluorescent protein,

under transcriptional control of a promoter active in *L. sakei* and transforming an host cell of *L. sakei* with said expression vector.

Preferably the expression vector is obtained from a replicative vector derived from a pG+host plasmid or from an integrative vector of the invention, allowing the stable insertion of an heterologous gene into the chromosomal *lacLM* operon. The reporter gene can be placed under transcriptional control of an exogenous promoter active in *L. sakei* or under transcriptional control of an endogenous *L. sakei* promoter. This second embodiment allows to study the regulation of said endogenous promoter under different environmental conditions.

The invention also includes detectable cells of *L. sakei*, obtainable by the above-defined process.

The invention can be used, for instance, to monitor the development of *L. sakei* in a complex medium, like in dry sausage fermentation, to estimate its competition with other flora, or to test new *L. sakei* starter strains in order to demonstrate their ability to develop in meat products.

These strains are thus good tools to monitor the development of *L. sakei* in environmental conditions applied in dry sausage fermentation and to estimate the competition with other flora. It should be possible to use both plasmids to test new *L. sakei* starter strains in order to demonstrate their ability to develop in meat products.

The present invention will be further illustrated by the additional description which follows, which refers to examples of expression of reporter genes in

L. sakei according to the invention. It should be understood however that these examples are given only by way of illustration of the invention and do not constitute in any way a limitation thereof.

EXAMPLES

Bacterial strains and growth conditions

Bacterial strains are listed in Table 1.

E. coli strain TG1 was used for cloning and subcloning experiments and for plasmid propagation. The *E. coli* strain GM2929 carrying a *dcm* mutation was used for the propagation of plasmids prior to digestions with the *BalI* restriction enzyme. The *E. coli* strain XL1Blue (Stratagene) was used for single strand DNA preparation.

E. coli TG1 were grown in Luria-Bertani (LB) medium [SAMBROOK *et al.*, Molecular cloning. A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, NY, (1989)] at 37°C with agitation.

L. sakei 23K isolated from sausage and plasmid cured was used as recipient strain for genetic constructions. *L. sakei* strains were grown at 30°C in the MRS medium [DE MAN *et al.*, Journal of Applied Bacteriology, 23, 130-135, (1960)]. For the inoculation in dry sausage model, *Staphylococcus carnosus* was grown at 30°C with agitation in the Brain Heart Infusion (BHI) medium (Difco). The Mannitol Salt Agar medium (MSA, Difco) was used for the detection of *S. carnosus* on dry sausage samples. The MCD medium was used for the detection of β-galactosidase activity in *lacZ* marked strain [LAURET *et al.*, Appl. Environ. Microbiol., 62, 1922-1927, (1996)].

L. sakei 23K and the GFP-marked strain RV1040 were inoculated at $2 \cdot 10^5$ cells/g and RV2012 was inoculated at $8 \cdot 10^3$ cells/g. *L. sakei* strains were inoculated together with *S. carnosus* 833 at 10^6 cells/g in dry sausage model prepared as described by MONTEL *et al.*, [Food Microbiology., 13, 489-499, (1996)]. Dry sausage models were incubated for 3 days at 22°C, then at 14°C. The survival of the strains at 0, 3, 11 and 28 days was followed by plating diluted aliquots on MRS and MSA.

Table I

Strains	Description	Reference or source
Lactobacillus sakei 23K	plasmid cured, recipient strain	A
RV1040	23K transformed with pRV85	this study
RV2002	23K, *lacL'Δ'lacM*	this study
RV1023	23 K, *lacL'Δ'lacM, atkY::lacZ*	this study
RV2011	23K, *lacLM*::pRV86	this study
RV2012	23K, *lacL'::pldhL::gfp$_{uv}$'lacM*	this study
Escherichia coli TG1	*supE, thi (lac-proAB), hsdD5, f'+ [traD36, proAB, lacI^{q}lacZΔM15]*	B

A : BERTHIER *et al.*, Microbiology (Reading)., 142, 1273-1279, (1996)
B: SAMBROOK *et al.*, Molecular cloning, A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, NY, (1989)

Electrotransformation procedures

E. coli and *L. sakei* electrocompetent cells were prepared and transformed by the methods of DOWER *et al.* [Nucleic acids research., 16, 6127-6145, (1988)] and BERTHIER *et al.* [Microbiology (Reading), (1996)], respectively. *E. coli* transformants were selected on LB agar plates containing ampicillin (100 mg·l^{-1}) or erythromycin (150 mg·l^{-1}). *L. sakei* transformants were isolated on MRS plates containing erythromycin (5 mg·l^{-1}).

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to the MRS agar medium at 32 mg·l^{-1} for the selection of *L. sakei* RV2012.

Plasmid isolation and characterization

Plasmids used and constructed in this study are listed in Table II. Plasmids were purified from *E. coli* cultures using the alkaline lysis method [BIRNBOIM and DOLY, Nucleic acids research., 7, 1513-1523, (1979)] or QIAGEN plasmid MAXIPREP kits. Plasmids from *L. sakei* were isolated following the method described by ANDERSON and McKAY [Applied and Environmental Microbiology, 46, 549-552, (1983)]. Chromosomal DNA was prepared by standard methods as previously described by STENTZ *et al.*, [Applied and Environmental Microbiology., 63, 2111-2116, (1997)]. Restriction and other DNA modifying enzymes were obtained from Boehringer Mannheim, and used as recommended by the manufacturer. Specific restriction fragments of DNA used in cloning experiments were extracted from agarose gels by the use of Gene Clean kit (Ozyme, France). Plasmids were restricted in such a way as to give compatible ends and were treated with alkaline phosphatase to prevent subsequent self-ligation.

Table II

Plasmids	Description	Reference or source
pRV300	Ap^R, Em^R non-replicative vector	A
$pGFP_{uv}$	Ap^R, source of gfp_{uv}	Clontech Laboratories
pRV80	Ap^R, Em^R, pRV300, *lacL'Δ'lacM* integrative vector	this study
pRV84	Ap^R, Em^R, pRV80 derivative lacking multiple cloning site	this study
pG+host5	Em^R, replicative vector	B
pRV85	Em^R, pG+host5, *pldhL::*gfp_{uv}	this study
pRV86	Ap^R, Em^R, pRV84 *pldhL::*gfp_{uv}	this study
pJM783	source of *lacZ*	C
pRV81	*atkY* cloned in front of *lacZ* in pJM783	this study
pRV83	Ap^R, Em^R, *atkY::lacZ* of pRV81 cloned in pRV300	this study

A: LELOUP *et al.*, Applied and Environmental Microbiology, 63, 2117-2123, (1997)
B: BISWAS *et al.*, Journal of Bacteriology, 175, 3628-3635, (1993)
C: PEREGO *et al.*, Molecular Microbiology, 2, 689-699, (1988)

EXAMPLE 1: CONSTRUCTION OF A VECTOR FOR INTEGRATION AT THE *lacLM* LOCUS OF *L. sakei*

The plasmid pRV80 was obtained by the cloning in pRV300 of two PCR amplified DNA fragments, containing respectively the 5'- and 3'-ends of *lacLM* with a large internal deletion. The *lacLM* operon of *L. sakei* DSM 20017 was previously cloned and sequenced [OBST *et al.*, Microbiology (Reading), 141, 3059-3066, (1995)].

Oligonucleotides deduced from this sequence were designed in order to amplify two fragments corresponding respectively to the 5'-end and 3'-end of *lacLM*. A restriction site (underlined) was added at the 5'-extremity of each primer.

lac1:
5'-GATCAAGCTTATGCTTTAAGGGTACTGG
lac2:
5'-ACGTGAATTCTTGTCATCGGACGTTGAA
lac4:
5'-GATCGAGCTCGCGCTTTGAACAATAGCT
lac6:
5'-ACGTGAATTCCGGTGCTGGATAATTGTT

A first PCR fragment, obtained with the lac1 and lac2 primers, was 513 bp long and contained the *lacLM* promoter and the 5'-end of *lacL*.

The second fragment, obtained with the lac4 and lac6 primers, was 522 bp long and contained the 3'-end of *lacM* and the downstream region. The two resulting PCR fragments were cloned in the pRV300 integrative vector.

In the resulting plasmid pRV80, the *lacLM* operon contains a 2193 bp internal deletion.

EXAMPLE 2: USE OF *LACZ* AS A REPORTER GENE

Construction of a *lacLM* mutant by double crossover, for use as recipient strain of *lacZ* fusions

In order to use *lacZ* as a reporter gene, it was necessary to first delete the *lacLM* operon of *L. sakei* 23K encoding the *L. sakei* β-galactosidase. Plasmid pRV80 can be integrated by two successive crossovers at the *lacLM* locus allowing gene replacement of the wild type *lacLM* operon by the deleted operon.

Plasmid pRV80 was used to transform *L. sakei* 23K for erythromycin resistance (see Fig.1).

The correct insertion of pRV80, by a Campbell-like recombination, at the *lacLM* locus was checked by a PCR experiment using the lac1 and lac4 primers. One transformant, RV2001, was then kept for further experiments. Since pRV80 contains the 5'-end and the 3'-end of the *lacLM* operon, its insertion in the chromosome by a single crossover in *lacLM* restored one copy of the wild-type operon and was therefore not mutagenic. Additionally, the insertion of pRV80 led to the duplication of part of the *lacLM* operon, a structure which is unstable unless selective pressure is maintained by the addition of erythromycin. In the absence of selective pressure, the plasmid can excise, by a second crossover, and is then lost since pRV300 can not replicate in *L. sakei*. Since one copy of the wild type *lacLM* operon, and one copy of the mutated *lacLM* operon are present after the first recombination, the excision of the plasmid could lead to the excision either of the wild type *lacLM* copy, or of the Δ*lacLM* copy. The excision of the mutated copy would restore a wild type genotype, whereas the excision of the wild type copy would lead to a copy of the *lacLM* operon with the internal deletion (Fig.1). In order to generate the second crossover, the RV2001 transformant was grown in MRS without erythromycin. After 100 generations, diluted culture aliquots were plated on MRS containing X-Gal and no erythromycin. Among 300 clones, two were white. The structure of the *lacLM* operon was verified by PCR on the chromosomal DNA extracted from these two clones, with primers complementary to various parts of *lacLM*. The expected 2.2 kb deletion of the internal part of *lacLM* was demonstrated. Moreover, these two clones were erythromycin sensitive and had thus lost the pRV300 moiety. This confirmed that the two strains were resulting from the expected recombination. One of the two clones was named RV2002 and was used for further constructions.

Figure 1 represents the steps of construction of an internal deletion in the *lacLM* operon. Transformants, selected on erythromycin can result from a crossover at position 1 or 2. In each case, one copy of the WT *lacLM* operon and one copy of the Δ*lacLM* operon are present. The duplication of part of the *lacLM* operon is unstable. By growing one transformant without erythromycin, strains resulting from a second crossover can be isolated. The plasmid can excise at position 3, leading to replacement of *lacLM* by Δ*lacLM*, or at position 4, restoring a WT genotype.

Insertion of *lacZ* under control of the inducible promoter of the *atkYB* operon

Isolation of the *atkYB* promoter and construction of an *atkY::lacZ* transcriptional fusion

The sequences located downstream from the *ptsI* gene of *L. sakei* [STENTZ *et al.*, Appl. Environ. Microbiol., 63, 2111-2116, (1997); and GenBank accession N° U82366] encode a putative copper efflux P-type ATPase and its negative regulator organized in an operon *(atkYB* operon). A 634 bp DNA fragment, containing the transcription terminator of *ptsI*, the putative promoter region of *atkYB*, and *atkY*, was amplified by PCR from chromosomal DNA of *L. sakei* using the following primers:

ATK1:

5'-GATCGAATTCTAGTCGAAGATTTTATGA, and

ATK3:

5'-GATCGGATCCCATCGTGTTTCATCGTTA

PCR experiments were performed on a Perkin-Elmer 9600 apparatus, with *Taq* DNA polymerase from Boehringer. Reactions were carried out in 100 µl mixtures containing 0.2 mM of each deoxynucleoside triphosphates, 1 µg chromosomal DNA template and 2.5 µM of each primer. Amplification was performed for 30 cycles (94°C, 1 min; 55°C, 2 min; and 72°C, 3 min).

Plasmid pJM783 contains the *lacZ* gene of *E. coli*, and a chloramphenicol resistance gene [PEREGO *et al.*, Molecular Microbiology, 2, 689-699, (1988)]. This plasmid has already been largely used to construct transcriptional or translational fusions in *Bacillus subtilis*. The 634 bp fragment was cloned in pJM783, upstream from *lacZ* resulting in pRV81.

pRV83 was obtained by cloning the *Eco*RI/*Bal*I fragment of pRV81 containing the *atkY::lacZ* fusion, into the integrative plasmid pRV300.

Chromosomal integration of the *atkY::lacZ* into the chromosome of *L. sakei*.

pRV83 was used to transform RV2002, deficient for β-galactosidase activity. The resulting strain, RV1023 contains an insertion of the *atkY::lacZ* fusion and an intact copy of the *atkYB* operon.

The β-galactosidase activity of RV1023 was tested on MCD plates containing X-Gal.

Fifty microliters of various solutions of metals or salts ions were added in the center of the plates and let to diffuse from the center to the periphery of the plates. Only copper allowed the expression of the *atkYB*::*lacZ* fusion as seen from the blue color of the clones. However the color was observed only at a certain distance from the center of the plates, suggesting that the expression was induced by a precise concentration of $CuSO_4$. Furthermore, the activity was higher when plates were incubated at room temperature (21 ± 1 °C) than at 30°C.

Further experimentations were performed in order to allow a more precise characterization of the β-galactosidase activity under induction by $CuSO_4$.

RV1023 was grown at 30°C in liquid MCD medium until OD_{600} 0.3, then various concentrations of $CuSO_4$ were added and cultures were incubated for 90 min at room temperature.

Bacteria from 10 ml culture aliquots were collected by centrifugation, and resuspended in 1 ml Z buffer (sodium phosphate, 100 mM, pH 7.0, KCl 10 mM, $MgSO_4$ 1 mM, β-mercaptoethanol 50 mM) containing 20% glycerol. Bacteria were broken with zirconium beads in a Fast-Prep bead beater (Bio 101), two times 20 sec at maximum speed with 5 min pause on ice. Cellular debris were removed by centrifugation. The β-galactosidase activity was measured in 1 ml Z buffer at 28°C. The reaction was started by the addition of 200 µl ONPG (4 mg ml^{-1}) and stopped with 500 µl Na_2CO_3 1M. Absorbance was measured at 420 nm. Activity was expressed in Miller Units [MILLER, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., p. 72-74, (1992)]. The results are the means of at least three independent assays.

The results of these experiments are shown in Figure 2, which represents the induction of β-galactosidase activity of the *atkYB*::*lacZ* fusion in RV1023 as a function of $CuSO_4$ concentration in the medium. These results show that, as expected from the observation made on plates, an optimal concentration of $CuSO_4$ exists that allows expression of the *atkYB*::*lacZ* fusion. This concentration, (30-40 µM) is much lower than the concentration required to inhibit the growth of *L. sakei*.

The transcription level of the *atkY*::*lacZ* fusion in different conditions allowed to identify the regulation of this operon and suggest that AtkB is a copper ATPase.

This study shows that *lacZ* can be used as reporter gene in *L. sakei*, and demonstrates that the 634 bp PCR amplified fragment comprises the copper

inducible promoter of *atkYB* which may be used in order to expressed foreign genes in *L. sakei*, with an expression controlled by the addition of small amounts of $CuSO_4$.

EXAMPLE 3: USE OF GFP AS A REPORTER GENE

In order to test GFP as marker expressed in *L. sakei*, two vectors were constructed: one replicative vector based on the plasmid pG+host5, and one integrative vector was derived from pRV80.

Construction of a replicative plasmid comprising the gfp_{uv} gene under control of the p*ldhL* promoter of *L. sakei*

A GFPuv protein (Clontech) optimized for the maximal fluorescence when excited by UV light was used. This modified GFPuv contains three amino acid substitutions (Phe99Ser, Met153Thr, Val163Ala), and fluoresces 18 times brighter than the wild-type GFP.

The gfp_{uv} gene was isolated from the plasmid pGFPuv (Clontech) as an *Hin*dIII/*Eco*RI fragment.

The promoter of the *ldhL* gene (p*ldhL*), encoding the L-LDH is known to be a strong constitutive promoter in *L. sakei* [MALLERET *et al.*, Microbiology, 144, 3327-3333, (1998)].

A 234 bp fragment encompassing this promoter was obtained by PCR amplification on *L. sakei* 23K chromosomal DNA. The primers used, designed from the known sequence of the *ldhL* gene (GenBank accession n° AF054624) were:

forward primer (corresponding to bases 3973-3990) ldh1:
5'-ATGCGAATTCTACTGAGAAGTTGCTCTC-3';
reverse primer (corresponding to bases 4190-4207) ldh2:
5'-AGCTAAGCTTTCGCCGACGAGGATAACT-3'.

Restriction sites added at the 5'-end of each primer are underlined.

The PCR amplification was performed on a Perkin-Elmer 9600 apparatus, for 25 cycles (94°C, 2 min; 55°C, 2 min; 72°C, 2 min) with a final elongation step at 72°C for 5 min. Reaction was carried out in 100 µl mix containing 1 µg chromosomal DNA template, 0.5 µM of each primer, 0.2 mM of each dNTP and 2.5 U of *Taq* DNA polymerase from Boehringer.

The amplified fragment was restricted by *Eco*RI and *Hin*dIII.

The p*ldhL* promoter and gfp_{uv} gene were cloned into pG+host5, at the *Eco*RI site. In the resulting plasmid, named pRV85, the gfp_{uv} gene is placed downstream from the constitutive promoter p*ldhL* (Fig. 3). The structure of pRV85 was verified by restriction analysis. The p*ldhL*::gfp_{uv} fusion was verified by DNA sequencing with the primers used for the amplification of the p*ldhL* promoter.

Plasmid stability

The replicative plasmid pRV85 was used to transform *L. sakei* 23K for erythromycin resistance. One transformant (RV1040) containing the plasmid was grown in liquid MRS medium without erythromycin in order to test the stability of the plasmid pRV85 in *L. sakei*. Approximately 10^3 bacteria were inoculated to100 ml of MRS, grown for 24 hours and then diluted to inoculate a new culture. In these growth conditions, cultures reached 10^8 bacteria/ml, which corresponds to approximately 20 generations per day. Plasmid survival was assessed during 100 generations by plating diluted aliquots on MRS medium with or without erythromycin and comparing duplicate colony counts on selective or non-selective MRS plates. After 100 generations under non-selective conditions, 100% of the clones were still erythromycin resistant, indicating that the plasmid pRV85 was still present. The presence of pRV85 was confirmed by fluorescence of clones and by restriction digestion of plasmids extracted from *L. sakei* colonies grown under non-selective conditions.

The stability of pRV85 in *L. sakei* 23K shows that this plasmid can be used as a valuable tool to maintain transformed strains even in the absence of antibiotic selection.

Emission of fluorescence

The GFP fluorescence intensity of the colonies of RV1040 grown at 14°C, 22°C and 30°C was quantitatively determined by the use of a FluorImager (Table III).

Table III

Growth temperature	Relative fluorescence intensity
30°C	471 ± 90
22°C	3868 ± 591
14°C	2398 ± 340

No fluorescence was detected in the control strain 23K. The maximum expression of GFP in RV1040 was obtained at 22°C. This expression was 1.5 fold and 8 fold weaker at 14°C and at 30°C, respectively, than at 22°C indicating a temperature dependent variation of the fluorescence of GFP.

Construction of an integrative plasmid comprising the *gfp*$_{uv}$ gene under control of the p*ldhL* promoter of *L. sakei*

In order to test whether *L. sakei* strains containing a single copy of the p*ldhL*::*gfp*$_{uv}$ fusion were detectable, the integrative plasmid pRV86 was also used to transform *L. sakei* 23K.

The plasmid pRV86 is a derivative of pRV80. First, the multiple cloning site, upstream from *lacL*, in pRV80 was deleted by digestion with *Bsp*120-I

and *Hin*dIII. The resulting plasmid, pRV84, presents a single *Eco*RI site between the *lacL* and *lacM* parts. An *Eco*RI fragment of pRV85 comprising the p*ldhL*::*gfp*$_{uv}$ fusion was cloned at the *Eco*RI site of pRV84, leading to pRV86 (Fig 3).

Chromosomal integration of the p*ldhL*::*gfp*$_{uv}$ into the chromosome of *L. sakei* 23K

The plasmid pRV86 which contains the fusion p*ldhL*::*gfp*$_{uv}$ flanked by 513 bp of the 5'-end of *lacL* and 522 bp of the 3'-end of *lacM* can be integrated into the chromosome of *L. sakei* by homologous recombination either in *lacL* or in *lacM*.

Figure 4 schematically represents the insertion of p*ldhL*::*gfp*$_{uv}$ in the *lacLM* operon of *L. sakei* 23K by two successive crossovers.

The *L. sakei* 23K strain was transformed with pRV86 for erythromycin resistance.

Single crossover integration of pRV86, at the *lacLM* locus, was checked by PCR on chromosomal DNA extracted from transformants. One transformant, RV2011, containing a single copy of pRV86 was selected and used for further experiments. This transformant, resulting from the integration of pRV86 by single cross-over, had one copy of the wild type *lacLM* operon and one copy of the mutated *lacLM* operon (Δ*lacLM* replaced by the p*ldhL*::*gfp*$_{uv}$ fusion , Fig. 5 A). In the absence of selective pressure, the plasmid can excise by a second, reverse, crossover. Since pRV86 is not replicative in *L. sakei*, the plasmid is lost after its excision. The second crossover could lead to the excision of the mutated Δ*lacLM* copy with the *gfp*$_{uv}$ gene or the wild-type *lacLM* copy. The excision of the Δ*lacLM*/*gfp*$_{uv}$ gene should restore a wild-type genotype, whereas the excision of the *lacLM* operon, should lead to gene replacement with a copy of the *gfp*$_{uv}$ gene, without any other heterologous DNA (Fig. 4 B). In order to generate the second crossover, the transformant RV2011 was grown in MRS without erythromycin. The presence of a wild-type *lacLM* operon can be detected by the blue color of colonies in the presence of X-Gal, reflecting β-galactosidase activity. After 100 generations, diluted culture aliquots were plated on MRS medium containing X-Gal. Among 400 clones, one was white suggesting that the *lacLM* operon was replaced by the p*ldhL*::*gfp*$_{uv}$ fusion. This clone, named RV2012, was erythromycin sensitive but still fluorescent. Chromosomal DNA from RV2012 was extracted and the structure of the integrated *gfp*$_{uv}$ gene was verified by PCR. As expected, the *lacLM* operon was replaced by the p*ldhL*::*gfp*$_{uv}$ fusion.

Emission of fluorescence

The GFP fluorescence intensity of the colonies of RV2012 grown at 14°C, 22°C and 30°C was determined in the same way as above for RV1040. The temperature dependent variation was similar to the one observed with RV1040.

At all temperatures, RV1040 exhibited a higher fluorescence intensity than *L. sakei* RV2012, which might reflect a higher expression of GFP because of the higher copy number of the p*ldhL*::*gfp*$_{uv}$ fusion in RV1040.

EXAMPLE 4: PROPERTIES OF GFP-MARKED *L. SAKEI* TRANSFORMANTS

Growth of the transformed strains

The putative burden caused by the presence of a plasmid in RV1040 or by the expression of GFP in RV1040 and RV2012 was examined. The control strain *L. sakei* 23K and the two transformants were grown in liquid MRS medium at 22°C, the incubation temperature for fermentation period of dry sausage, and 30°C, the optimal growth temperature for *L. sakei*.

Figure 5 represents the growth of the wild type strain 23K (squares), the strain RV1040 transformed with replicative plasmid pRV85 (triangle) and the strain RV2012 containing an integrated copy of *gfp*uv (circles) grown at 22°C (open symbols) or 30°C (bold symbols).

At both temperatures, no difference was detected between the parental strain 23K and the two transformed strains. This shows that no excessive metabolic burden was caused by the presence of the constructions.

Lactate production

In both transformants, a single copy of the *ldhL* gene is present but its promoter is duplicated. The amount of L-lactate in culture supernatants of 23K and the two transformed strains RV1040 and RV2012 was measured with a Boehringer Mannheim kit, in the conditions described by the manufacturer.

Figure 6 represents the L-lactate concentration in culture supernatants of the strains 23K, RV1040 and RV2012 grown in MRS medium for 10 or 24 hours at 22°C (open symbols) or 30°C (bold symbols).

The L-lactate concentration produced by RV1040 and RV2012 was similar to that produced by the wild-type strain. The production of L-lactate was thus not affected by the presence of the *gfp*$_{uv}$ gene under the control of *ldhL* promoter. For the three strains, the pH of the culture supernatants after 24 hours culture dropped from 5.6 in the initial medium to 4.2 at 30°C and 4.4 at 22°C.

Monitoring GFP-marked *L. sakei* in dry sausage

Since the expression of GFP was detectable in both GFP-marked *L. sakei* transformants, these strains were used in a complex environment.

GFP-marked *L. sakei* strains were inoculated in dry sausages with *S. carnosus* 833, and assessed during 28 days (Fig. 7). Bacterial flora was followed by

plating diluted aliquots on MRS and MSA media, and by the detection of fluorescence.

Figure 7 represents the bacterial counts (cfu/g) observed after plating of dry sausage sample aliquots on MRS plates. The dry sausage samples were inoculated with 23K (squares), RV1040 (triangles) or RV2012 (circles).

In all dry sausage samples, the number of lactic acid bacteria increased during the first 3 days (from $8 \cdot 10^3$ or $2 \cdot 10^5$ to $4\text{-}7 \cdot 10^8$ cfu/g), then stabilized at $4\text{-}7 \cdot 10^8$ cfu/g during 28 days.

The implantation of RV1040 and RV2010 was assessed by measuring the percentage of fluorescent colonies.

To detect GFP-tagged *L. sakei* strains directly in dry sausages models, samples were diluted in saline solution (NaCl 8,5 $g \cdot l^{-1}$) and stomached for 1 min. For the observation of fluorescent cells by epifluorescence microscopy the cells were prepared as follows: liquid cultures or dry sausage suspensions were centrifuged, cells were washed in saline solution and smeared on microscope slides. Before observation, slides were treated by overlaying with CITIFLUOR.

An epifluorescent microscope (ZEISS) equipped with a GFP filter set (Excitation 470 nm; emission 505-530 nm) was used to visualize fluorescent cells. To quantify the fluorescence of bacterial clones, colonies on MRS plates were observed by using a FluorImager (Molecular Dynamics) coupled with an image analysis software (Image Quant).

The isolates from MRS plates of dry sausage samples inoculated by the 23K strain, exhibited no fluorescence. On the contrary, 100% of colonies isolated from samples inoculated by strains RV1040 and RV2012 were fluorescent at 0, 3, and 11 days. At 28 days, 100% of the clones, isolated on MRS, from sausages inoculated with RV2012 were fluorescent whereas 95% of fluorescent clones were detected with sausages inoculated with RV1040. Fluorescent strains were assimilated to *L. sakei* RV1040 and RV2012. The non fluorescent clones, isolated from dry sausage samples inoculated by RV1040, could correspond to lactobacilli of natural flora, or to the loss of the marker plasmid pRV85 in *L. sakei* RV1040.

Although pRV85 was shown to be extremely stable after 100 generations in MRS medium, the plasmid might be less stable, or the strain RV1040 carrying the plasmid might grow more slowly in a complex medium such as dry sausage. When inoculated at $2 \cdot 10^5$ cfu/g with RV1040, the final counts measured in dry sausage samples reached $7 \cdot 10^8$ cfu/g which corresponds to approximately 12 generations, far less than the 100 generations followed in laboratory growth conditions. The GFP-marked *L. sakei* strains RV1040 and RV2012 can nevertheless be detected directly in dry sausage suspension by epifluorescence microscopy.

CLAIMS

1) An integrative vector resulting from the insertion of a portion of the *lacLM* operon of *L. sakei* comprising at least 300 pb of the 5'-end of *lacL* and at least 300 pb of the 3'-end of *lacM* of into vector pRV300.

2) An integrative vector of claim 1 further comprising an heterologous DNA sequence inserted between the *lacL* and the *lacM* sequences.

3) An integrative vector of claim 2, wherein said heterologous DNA sequence is selected among:

- a gene encoding the β-galactosidase of *E. coli*, and
- a gene encoding the green fluorescent protein.

4) A process for obtaining a stable transformant of *L. sakei* wherein said process comprises transforming a *L. sakei* host cell with a vector selected among:

- - an integrative vector of any of claims 1 to 3;
- - a derivative of a pG+host plasmid.

5) A transformant of *L. sakei* obtainable by the process of claim 4.

6) A transformant of claim 5, wherein said transformant expresses the green fluorescent protein.

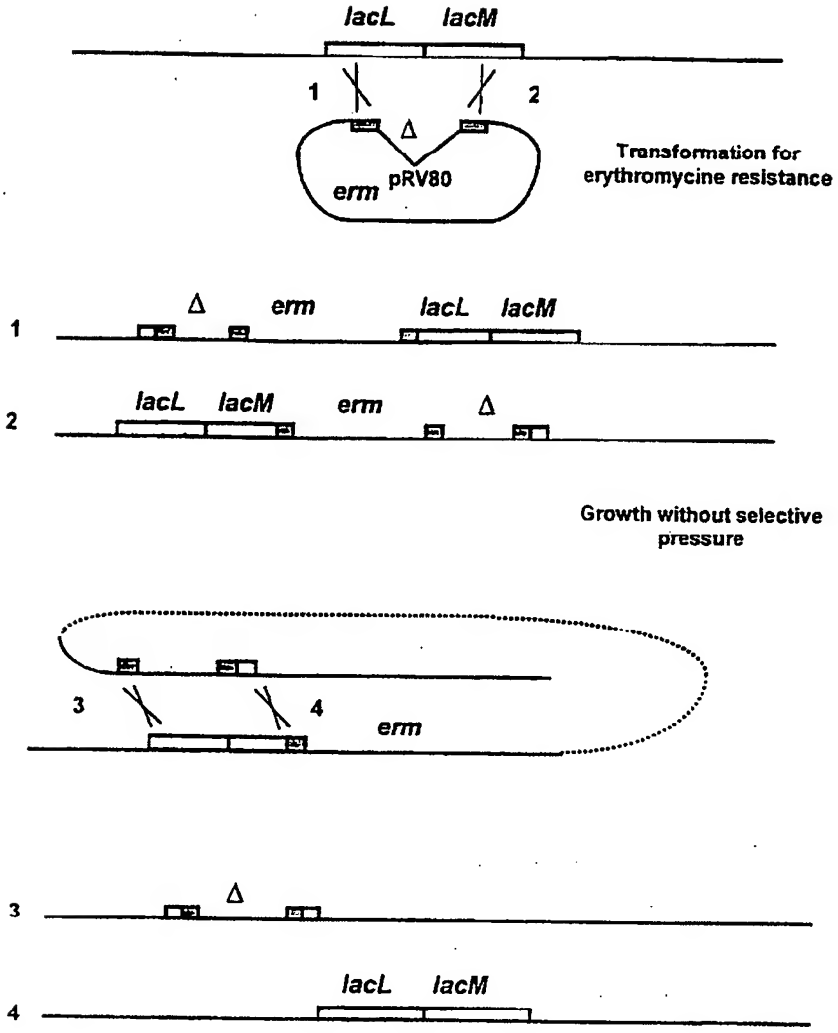

lacL
lacM
1
2
Δ
erm
pRV80
Transformation for erythromycine resistance
1
Δ
erm
lacL
lacM
2
lacL
lacM
erm
Δ
Growth without selective pressure
3
4
erm
3
Δ
4
lacL
lacM

FIG. 1

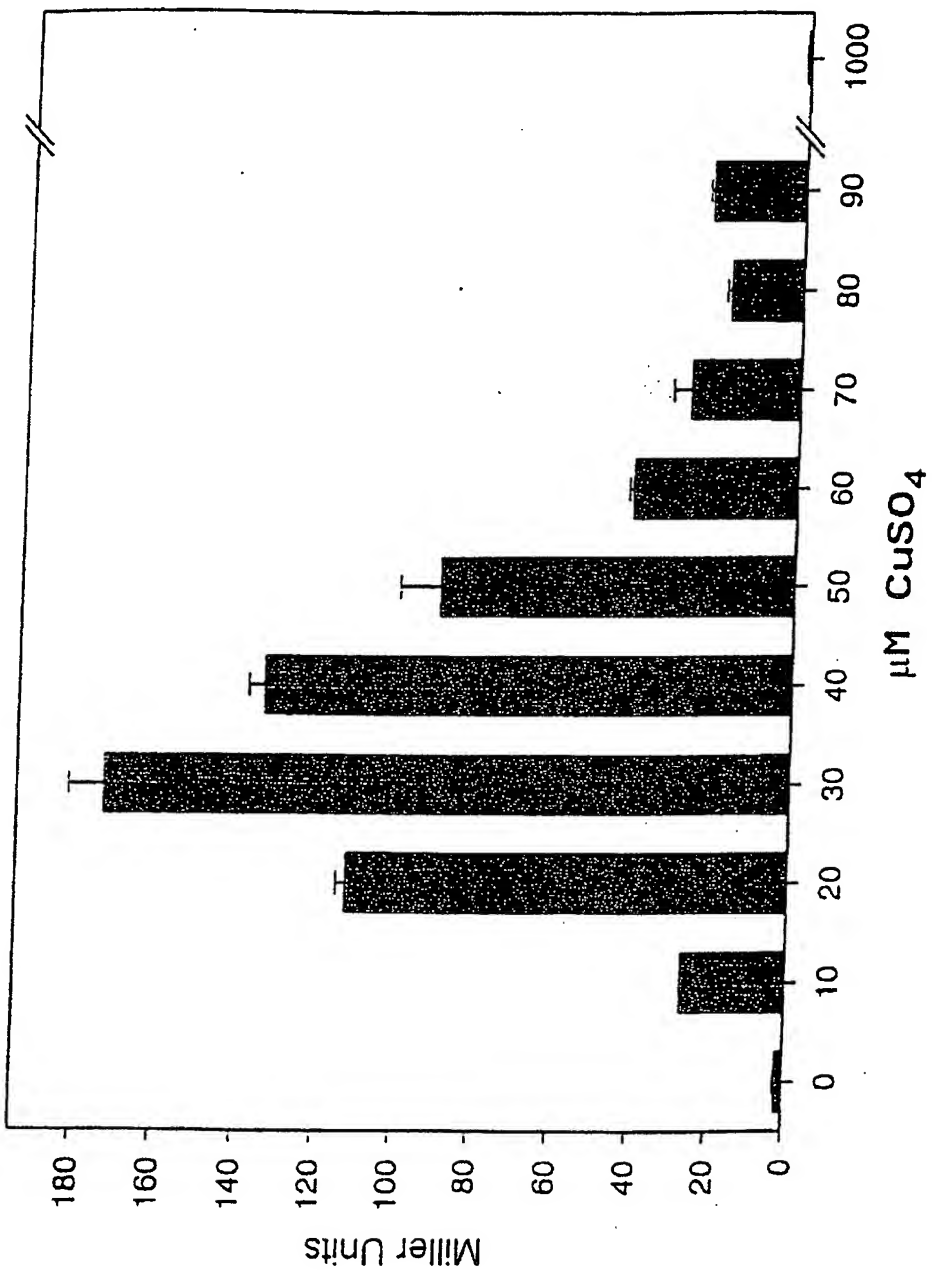

Miller Units
0
20
40
60
80
100
120
140
160
180
0
10
20
30
40
50
60
70
80
90
1000
µM CuSO4

FIG. 2

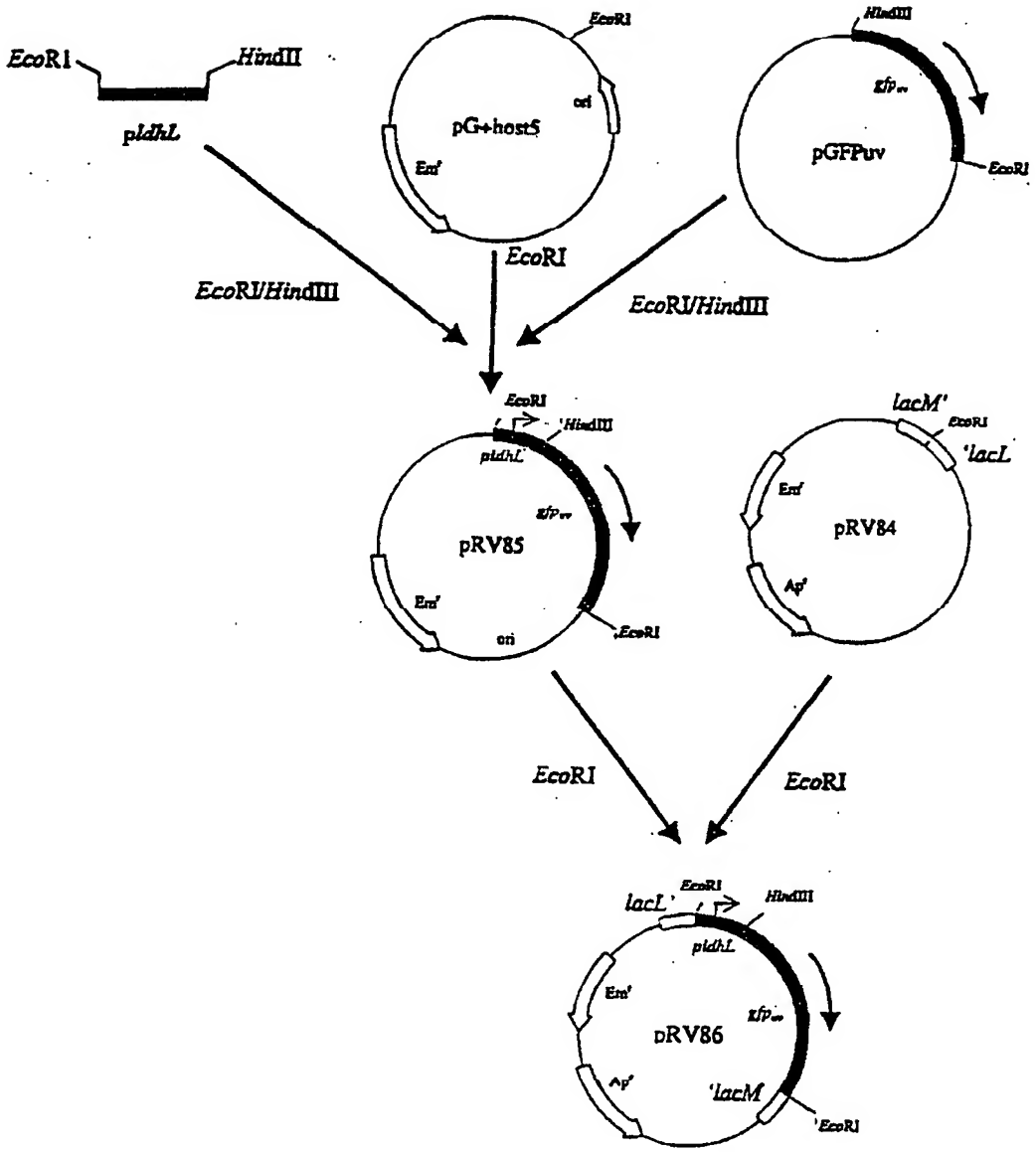

EcoR1
HindIII
pldhL
pG+host5
ori
Ery'
EcoRI
EcoRI/HindIII
EcoRI/HindIII
HindIII
Zfp-uv
pGFPuv
EcoRI
EcoRI
EcoRI
'HindIII
pldhL
Zfp-uv
pRV85
Ery'
ori
EcoRI
lacM'
EcoRI
'lacL
Ery'
pRV84
Ap'
EcoRI
EcoRI
lacL'
EcoRI
HindIII
pldhL
Ery'
Zfp-uv
pRV86
Ap'
'lacM
EcoRI

FIG. 3

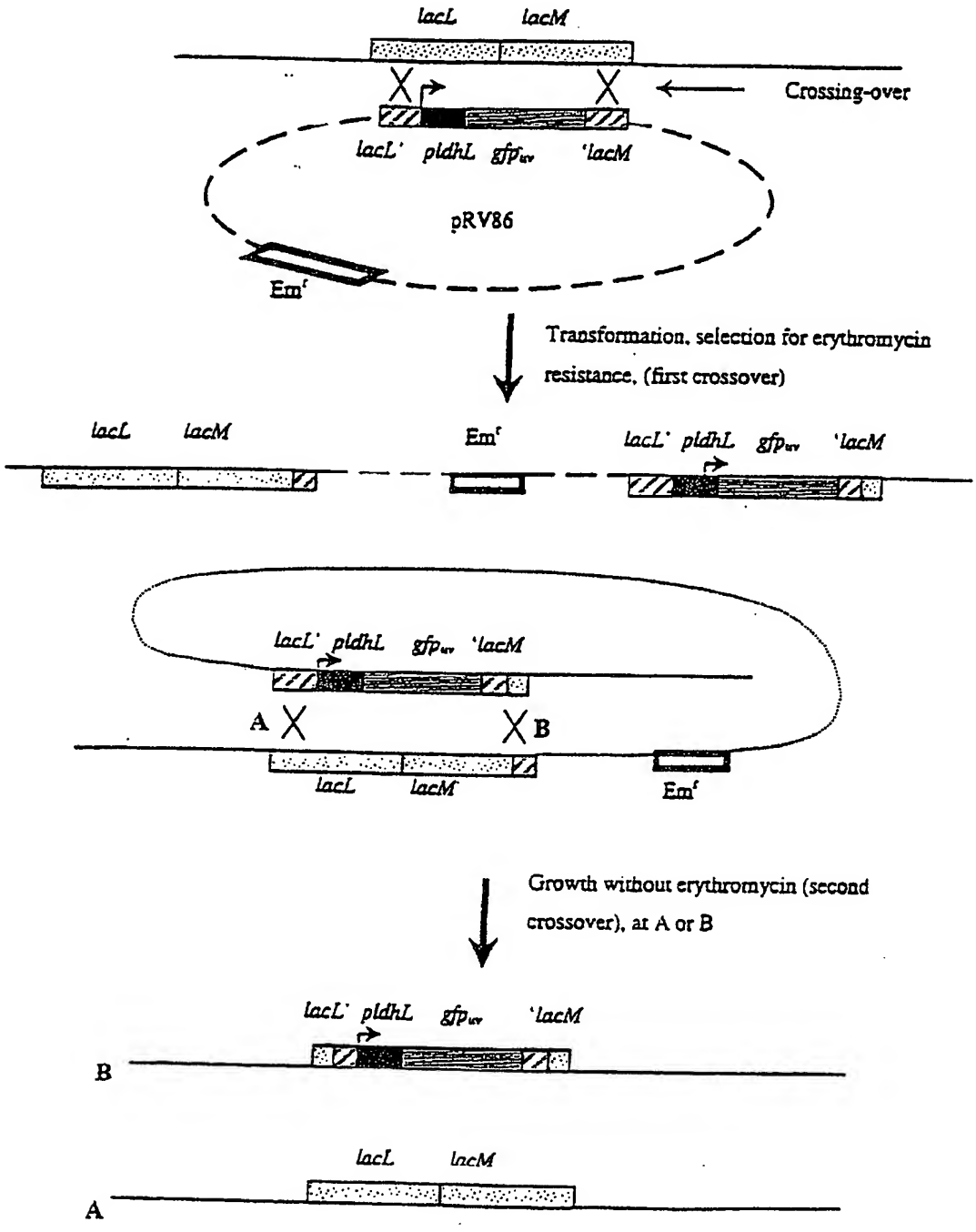

lacL
lacM
Crossing-over
lacL' pldhL gfpuv 'lacM
pRV86
Emr
Transformation, selection for erythromycin resistance, (first crossover)
lacL
lacM
Emr
lacL' pldhL gfpuv 'lacM
lacL' pldhL gfpuv 'lacM
A
B
lacL
lacM
Emr
Growth without erythromycin (second crossover), at A or B
lacL' pldhL gfpuv 'lacM
B
lacL
lacM
A

FIG. 4

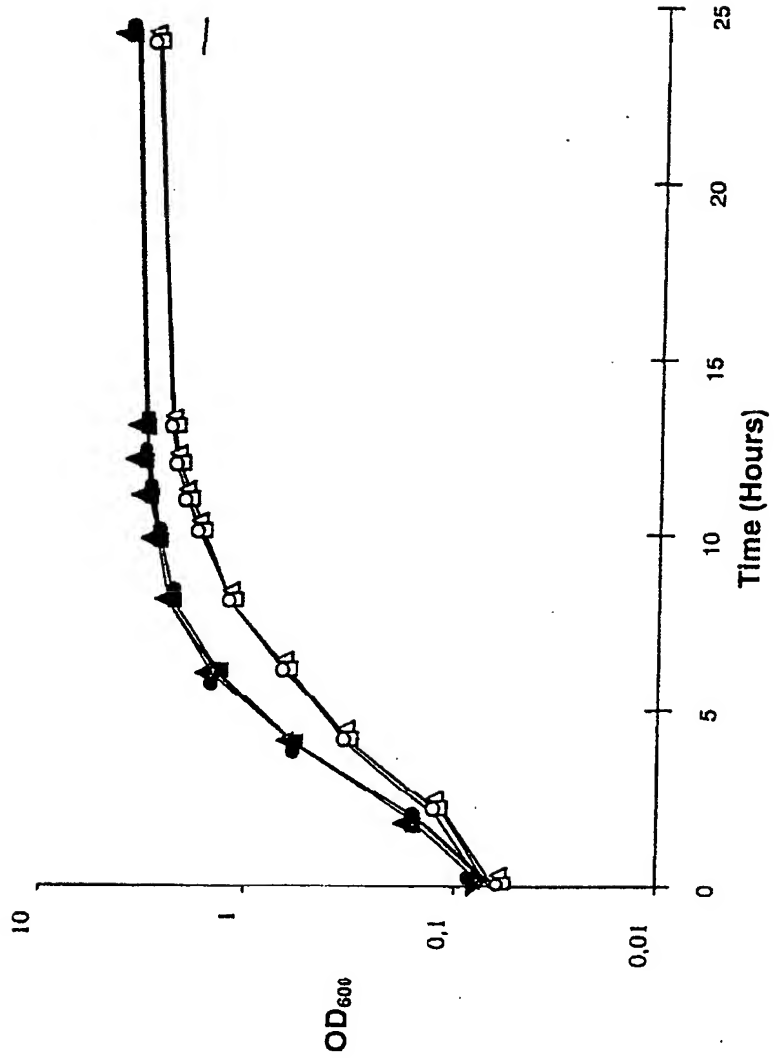


FIG. 5

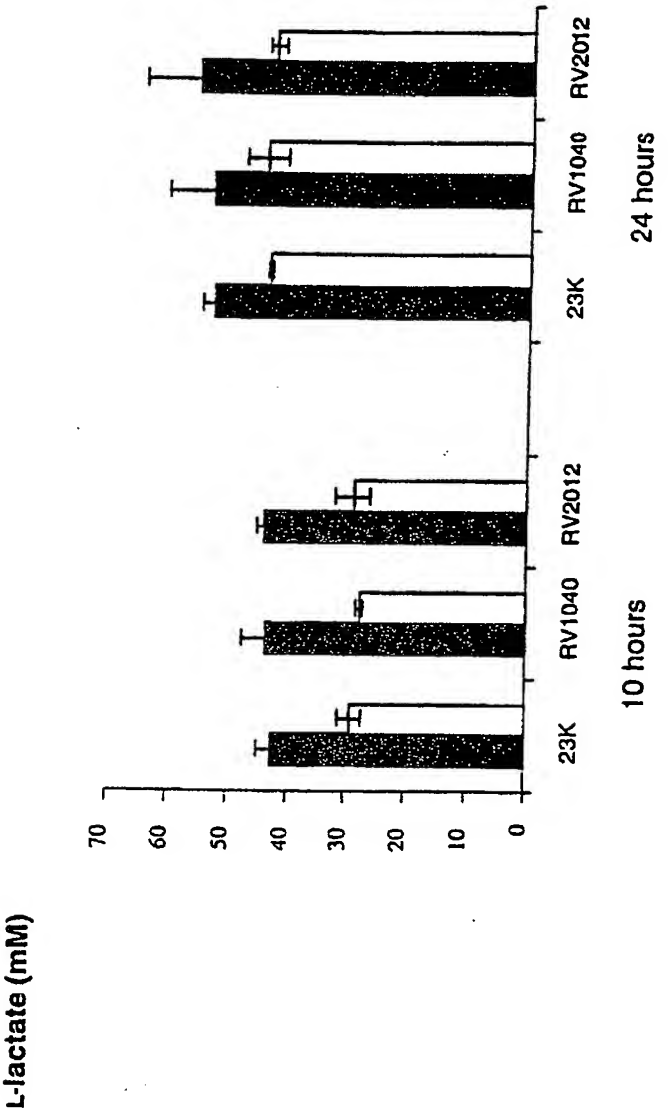

L-lactate (mM)
70
60
50
40
30
20
10
0
23K
RV1040
RV2012
10 hours
23K
RV1040
RV2012
24 hours

FIG. 6

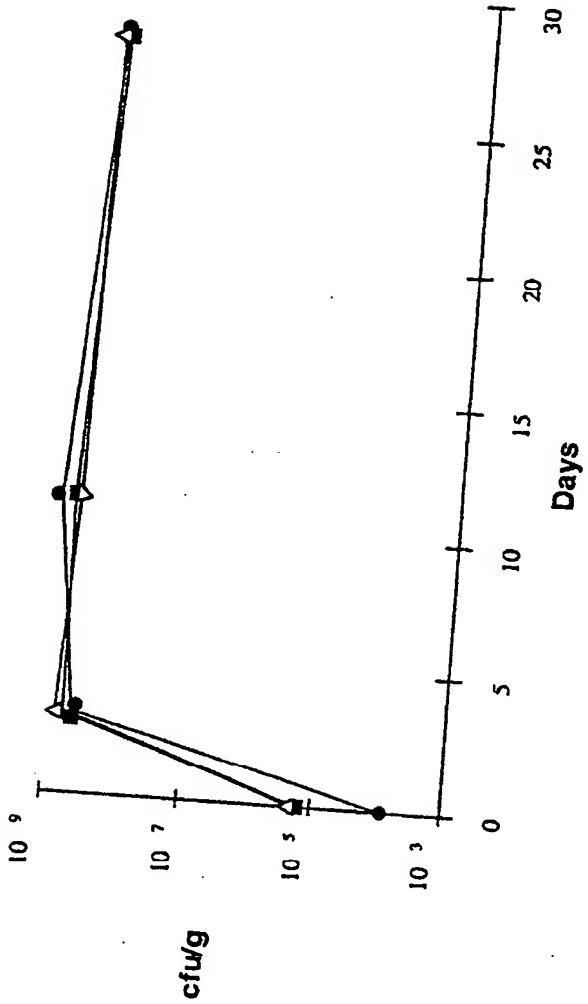


FIG. 7

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/EP 00/03099

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/74 //C12R1/225

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FR 2 770 536 A (TEXEL) 7 May 1999 (1999-05-07) page 10, line 12 -page 11, line 8 page 13, line 19 -page 16, line 25 figure 1 ---	1-6
A	LELOUP LAURENCE ET AL: "Single-crossover integration in the Lactobacillus sake chromosome and insertion inactivation of the ptsI and lacL genes." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 6, 1997, pages 2117-2123, XP002159436 ISSN: 0099-2240 cited in the application page 2120, left-hand column, last paragraph -page 2121, right-hand column, paragraph 3 --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 February 2001	09. 5. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ALCONADA RODRIG.., A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna tl Application No
PCT/EP 00/03099

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	STENTZ REGIS ET AL: "Development of genetic tools for Lactobacillus sakei: Disruption of the beta-galactosidase gene and use of lacZ as a reporter gene to study regulation of the putative copper ATPase, AtkB." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 66, no. 10, October 2000 (2000-10), pages 4272-4278, XP000980455 ISSN: 0099-2240 -----	1-6

5

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int tional application No.
PCT/EP 00/03099

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,2,3 (complete) and 4,5,6 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1,2,3 (complete) and 4,5,6 (partially)

An integrative vector resulting from the insertion of a portion of the lacM operon of L.sakei comprising at least 300 bp of hte 5'-end of lacL and at least 300 bp of the 3'-end of lacM into vector pRV300; said integrative vector further comprising an heterologous DNA sequence inserted between the lacL and lacM sequences; wherein said integrative vector encodes E.coli beta-galactosidase or green fluorescent protein; a process for obtaining a stable transformant of L.sakei comprising transforming a L.sakei with said integrative vector; a transformant L.sakei obtainable by the process of claim 4 and said transformant which expresses the green fluorescent protein.

2. Claims: 4,5,6 (partially)

A process for obtaining a stable trasformant of L.sakei wherein said process comprises transforming a L.sakei host cell with a derivative of a pG+host plasmid, a transformant obtainable by said process and a transformant which expresses green fluorescent protein.

INTERNATIONAL SEARCH REPORT

.rmation on patent family members

Interna l Application No
PCT/EP 00/03099

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR 2770536 A	07-05-1999	AU 1158799 A	31-05-1999
		EP 1029063 A	23-08-2000
		WO 9924591 A	20-05-1999
		PL 340709 A	26-02-2001